ELSEVIER

Journal of Cystic Fibrosis 11 (2012) 398–404

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Original Article

GUSB and *ATP2B4* are suitable reference genes for *CFTR* gene expression data normalization in nasal epithelium cells

Laia Masvidal [a], Antoni Alvarez [b], Laura Ruano [b], Javier de Gracia [b], Sara Larriba [a], Teresa Casals [a,*]

[a] *Human Molecular Genetics Group, IDIBELL, 08908 Barcelona, Spain*
[b] *Cystic Fibrosis Adult Unit, Hospital Vall d'Hebron, 08035 Barcelona, Spain*

1 December 2011; 14 February 2012; 21 March 2012
Available online 22 April 2012

Abstract

Background: *CFTR* expression studies contribute in understanding the relationship between *CFTR* transcripts and clinical outcomes. Normalization of qPCR data is an essential step to determine target gene expression. Consequently, appropriate reference genes must be selected for each gene/tissue. In this work, we have assessed the suitability of four potential reference genes for *CFTR* expression analysis in nasal epithelium.
Methods: *B2M*, *GUSB*, *HPRT1* and *ATP2B4* expression was evaluated in nasal epithelium samples (*CFTR*-wt controls, n=21; *CFTR*-splicing group, n=18) by RT-qPCR. Calibration curves were built and different analyses (geNorm, NormFinder, Mann–Whitney) were performed to evaluate gene expression stability between samples as well as between groups.
Results and conclusions: We have applied an accurate approach to select reference genes for *CFTR* expression analysis in nasal epithelium. From the four genes assessed, *GUSB* and *ATP2B4* have been validated as a reliable gene combination for *CFTR* gene qPCR data normalization.

Keywords: Cystic fibrosis; *CFTR*; Gene expression; Nasal epithelium; Reference gene; RT-qPCR

1. Introduction

Cystic fibrosis (CF; OMIM 219700) is a multi-systemic disease caused by *CFTR* gene mutations (*ABCC7*; OMIM 602421) which mainly affect ion transport at the epithelial tissues. Transcript amount has been associated with a broad phenotypic spectrum from CF to *CFTR*-related disorders [1]. To determine the clinical relevance of *CFTR* expression level, several approaches have been developed providing transcript quantification of normal and mutant alleles in different tissues [2–5].

In recent years, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) has become increasingly relevant because of its capacity to determine the expression level of a target gene in different sample types [6]. Its simplicity, specificity and sensitivity make RT-qPCR the most suitable quantitative method for gene expression analysis [7,8]. However, some factors related to biological and technical variability, such as differences in RNA starting material, PCR efficiency, reverse transcription enzymatic efficiency or different tissue and cell transcriptional activity, may lead to serious misinterpretation of results [9–11]. Regarding these variables, recent guidelines have been published to minimize their effect [7].

In particular, the use of reference genes is commonly accepted as the most appropriate strategy for data normalization [6,12,13]. An ideal reference gene must be stably expressed, non-regulated and not affected by biological or experimental conditions [14]. So far, the published data suggest that there are

* Corresponding author at: Human Molecular Genetics Group, Bellvitge Biomedical Research Institute (IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199, 08908 Barcelona, Spain. Tel.: +34 93 260 74 25; fax: +34 93 260 74 14.
E-mail address: tcasals@idibell.cat (T. Casals).

1569-1993/$ -see front matter
doi:10.1016/j.jcf.2012.03.008

no genes whose expression is universally stable across all types of tissue and/or condition [11]. Therefore, it is critical to perform a previous evaluation study aimed at identifying the most appropriate reference genes for each target gene and experiment.

Currently, most of the *CFTR* cDNA analysis is performed on nasal epithelial (NE) cells, due to their *CFTR* expression and easy harvesting. In this study, we have evaluated four candidate reference genes to select the most reliable ones to normalize *CFTR* qPCR data for gene expression studies in NE cells. The major challenges of this work have been the low *CFTR* gene expression and the limited amount of NE cells for *ex vivo* analysis.

2. Material and methods

2.1. Samples

Three sample types were analyzed in this study: 1) The Human Embryonic Kidney 293 cell line over-expressing wild type *CFTR* cDNA (HEK 293-*CFTR*); 2) a nasal polyp (NP) from a p.Phe508del homozygous CF patient; and 3) NE samples from 39 adult individuals, *CFTR*-wild type controls (n=21) and *CFTR*-splicing group (n=18). All individuals in the second group bore the same class V splicing mutation associated with CF. Class V mutations show a lower amount of full length transcripts and differences in expression level have been reported concerning different class V mutations [2–5]. Consequently, full length *CFTR* transcripts are expected to be lower in the *CFTR*-splicing group. No other significant differences were expected as p.Phe508del causes a misfolded protein (class II). All participants gave their written consent and the study was approved by the local ethics committee.

HEK 293 cell line culture and cell transfection with the pCMVCFTRNot6.2 expression vector carrying the 6.2 kb human wild type *CFTR* cDNA were carried out as previously described [15]. Cells were collected in RNA lysis buffer (Stratagene Agilent Technologies, Waldbronn, Germany) 48 h post-transfection and stored at −20 °C until processing.

NP was excised and frozen in liquid nitrogen immediately. NE cells were collected using a cytology brush (Eurogine S.L., Spain). Brushes were placed directly in 1 ml RNAlater buffer (Qiagen, Hilden, Germany). RNAlater maintains RNA integrity and allows a homogeneous process for all samples while stored at 4 °C overnight, and frozen at −80 °C until processing.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted using Absolutely RNA Miniprep Kit (Stratagene Agilent Technologies, Waldbronn, Germany), according to manufacturer's instructions. Samples were treated with DNase to prevent genomic DNA interference. RNA quantity and quality were first measured in a NanoDrop ND-100 (Nanodrop Technologies, Montchanin, DE, USA). Furthermore, the integrity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) which provides the RNA integrity number (RIN) (scores range from 10 to 1, the former being the highest RNA quality) [16], and 260/280 nm ratio values. To reduce RNA variability from NE samples, we established several threshold inclusion parameters: a) RNA concentration ≥ 50 ng/μl; b) 260/280 nm ratio ≥ 1.90; and c) RIN ≥ 5. No significant differences in RIN values were found between groups (*CFTR*-wild type 6.12±0.78, *CFTR*-splicing 6.10±0.61, $p=0.90$).

For cDNA synthesis, RNA samples were denatured for 5 min at 65 °C and cooled on ice. RNA samples (500 ng) used in this study were simultaneously reverse transcribed (RT) in a 20 μl reaction mixture using the High-capacity cDNA reverse transcription kit and random primers (Applied Biosystems, Foster City, CA, USA) according to the protocol. Duplicated RT reactions were carried out for each sample. In addition, a pool of all control samples was additionally obtained as an extra sample before RT to create calibration curves. cDNAs were stored at −20 °C until RT-qPCR experiments were performed.

2.3. Candidate reference genes

Four candidate reference genes were selected from the literature: *Beta-2-microglobulin (B2M), Glucuronidase-beta (GUSB), Hypoxanthine phosphoribosyltransferase 1 (HPRT1)* and *Plasma membrane Ca^{++}-transporting ATPase 4 (ATP2B4; PMCA4)*. It is worth noting that, unlike commonly used reference genes, we have included in this study the *ATP2B4* gene [17] encoding a membrane protein as *CFTR* does. The four genes have independent functions in cellular maintenance and, regulation of their expression is assumed not to be directly related (Table 1).

2.4. qPCR

qPCR analysis was performed on an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using pre-designed cDNA specific Taqman™ assay-on-demand products (Table 1). Reactions were carried out in a total volume of 20 μl containing 10 μl Universal Master Mix No UNG 2× (Applied Biosystems, Foster City, CA, USA), 1 μl Taqman™ assay (20×), 8 μl RNase-free water and 1 μl cDNA. PCR universal conditions were, 10 min template denaturation at 95 °C and 40 cycles with a denaturation at 95 °C for 15 s, and a combined primer annealing/elongation at 60 °C for 1 min. Triplicate reactions were carried out for each sample/gene combination. No RT and no RNA samples were included to test for PCR and genomic DNA contamination, respectively. Furthermore, a calibrator sample was included in every run to correct for inter-run variability.

Four points of a 5-fold serial dilution were created from the control pool sample and used to build a calibration curve for each gene. Triplicate reactions were also carried out. For each calibration curve a correlation coefficient was generated (R^2) and used as a goodness of fit. Amplification efficiency was obtained using the following equation: $E=10^{(-1/slope)}$, where E is the PCR efficiency and "slope" is the slope of the regression line generated. An E value of 2 corresponds to the maximum

Table 1
The four candidate reference genes and *CFTR* target gene characteristics.

Gene name	Symbol	Accession num.	Function	Location	Assay [a]
Beta-2-microglobulin	*B2M*	NM_004048	Beta-chain of major histocompatibility complex class I molecules	15q21-q22.2	Hs00984230_m1
Glucuronidase, beta	*GUSB*	NM_000181	Glycosaminoglycans degradation	7q21.11	Hs99999908_m1
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	NM_000194	Purine synthesis in salvage pathway	Xq26.1	Hs01003267_m1
ATPase, Ca^{++} transporting, plasma membrane 4	*ATP2B4*	NM_001684	Calcium transmembrane transport	1q32.1	Hs00608066_m1
Cystic fibrosis transmembrane conductance regulator	*CFTR*	NM_001104950	Chloride transmembrane transport	7q31.2	Hs00357011_m1

[a] ABI gene expression assay ID (Applied Biosystems).

efficiency. The raw quantification cycle (Cq) values were determined using SDS software v1.3.1 (Applied Biosystems, Foster City, CA, USA) and a manual threshold among triplicates was applied for all genes at 0.200.

To confirm reproducibility and precision of real time experiments, intra-assay and inter-assay variation from the raw Cq values were determined for all samples (n=39). Variation was measured as the coefficient of variation (CV) of Cq values. The CV intra-assay ranges were 0.04–1.05 (RT1) and 0.06–1.08 (RT2) for *GUSB*; 0.03–1.05 (RT1) and 0.05–1.18 (RT2) for *ATP2B4*; and 0.05–0.78 (RT1) and 0.05–1.21 (RT2) for *HPRT1*, covering over 90% of the analyzed samples. In addition, the CV inter-assay (RT1 *vs.* RT2) ranges were 0.01–3.81 for *GUSB*, 0.11–2.63 for *ATP2B4* and 0.12–5.73 for *HPRT1*, in the 90% of samples (Fig. 1).

For each RT-qPCR experiment, Cq values were converted to relative quantities (RQ) and subsequently to calibrated normalized relative quantities (CNRQ) using qBase^{Plus} software (Biogazelle NV, Zwijnaarde, Belgium) [18]. Its algorithm uses gene-specific amplification efficiency values and allows normalization with multiple reference genes. Mean values from both RT-qPCR for each sample was considered for statistical evaluation.

2.5. Statistical analysis

A Mann–Whitney test using SPSS 12.0 (SPSS Software, Chicago, IL, USA) was applied to evaluate differential expression between groups. Differences were considered statistically significant when $p<0.05$.

To assess the stability of candidate reference genes two different softwares, geNorm and NormFinder, were applied. GeNorm software [9] calculates the gene expression stability value M as the average pair wise variation of a particular gene compared with all other candidate reference genes (RQ values). Additionally, geNorm also determines the optimal number of genes to an accurate normalization. All genes with an M value<0.5 are considered stable. A coefficient of variation (CV) from normalized data, is also determined providing a measure of expression dispersion (cutoff value<0.200). In the NormFinder program [19] the stability value is based on the combined estimate of intra- and inter-group expression variations (Cq values) for each candidate reference gene. A low stability value indicates a low combined intra and inter-group variation and proves high expression stability. Using this measure, the most stable gene and the best combination of two genes for data normalization are calculated.

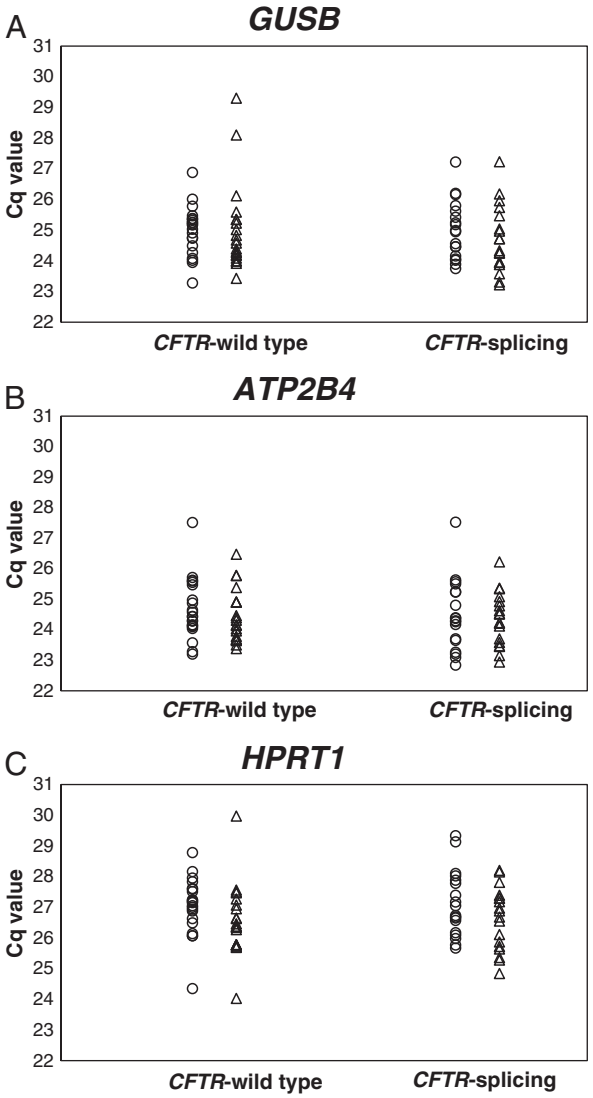

Fig. 1. Absolute expression values of the three reference genes in all the samples of the study. Mean values: A) *CFTR*-wild type RT1=24.98, RT2=25.00; *CFTR*-splicing RT1=25.01, RT2=24.71; B) *CFTR*-wild type RT1=24.69, RT2=24.42; *CFTR*-splicing RT1=24.48, RT2=24.35; C) *CFTR*-wild type RT1=27.04, RT2=26.54; *CFTR*-splicing RT1=27.12, RT2=26.62. RT1 (circle); RT2 (triangle).

3. Results

3.1. Absolute gene expression

We analyzed the transcript abundance of all four genes in the three different cell types, HEK 293-*CFTR,* NP and NE (pool of control samples), by direct comparison of their Cq values. The PCR efficiency and the expression profile were determined for each single gene assay using the calibration curves (Table 2). Efficiency for each candidate reference gene ranged from 1.853 to 1.901 and their correlation coefficients (R^2) ranged from 0.986 to 0.997.

The *B2M* gene showed a similar expression profile in HEK 293-*CFTR* and NP. In addition, when the *B2M* expression profile was compared with the corresponding expression of remaining genes in NP, it showed the highest expression level (*i.e.*, 16.0–26.4 *vs.* 26.4–34.5 in *CFTR*). Such different expression behavior was considered enough to exclude *B2M* for further analysis. As expected, *CFTR* gene expression was higher in HEK 293-*CFTR* compared to NP and NE, because of its *CFTR* over-expression. The comparison of the Cq values between nasal tissues resulted in a similar expression level (*GUSB*, *ATB2B4*) or lower (*HPRT1*, *CFTR*) in NE than in NP (Table 2).

3.2. Candidate genes analysis

First, the mean value of raw Cq values from RT1 and RT2 were analyzed in a pilot study, *CFTR*-wt controls (n=7) and *CFTR*-splicing group (n=9), using the Mann–Whitney test, which showed no statistically significant differences between groups for the three candidate genes ($p=0.606$ for *GUSB* and *ATP2B4*; $p=0.408$ for *HPRT1*).

Then, gene stability was assessed by two different programs. The NormFinder software ranked *HPRT1* as the most stable gene (0.083) and *HPTR1* and *ATP2B4* as the best combination of two genes for target gene data normalization (0.093). Individually, *GUSB* and *ATP2B4* presented a Normfinder stability value of 0.150 and 0.153, respectively.

On the other hand, the geNorm software showed an M value higher than 0.5 for all three genes analyzed (*GUSB*, 0.571; *ATP2B4*, 0.596; *HPRT1*, 0.787). CV analysis that resulted from the application of this software also determined that *GUSB* and *HPRT1* were above the cutoff value, 0.200 and 0.453 respectively. Therefore, the combination of the three genes was considered unstable. However, when the gene showing greater M value (*HPRT1*) was omitted from the analysis, the combination of the two other genes was considered stable, showing M and CV value into the range, M=0.354 CV=0.128 for *GUSB*; M=0.354 CV=0.119 for *ATP2B4*.

The Mann–Whitney test showed no statistically significant differences between groups for any of the genes studied, when the data were normalized with either the combination of three genes (*GUSB*, 0.96±0.07 *vs.* 0.86±0.06, $p=0.090$; *ATP2B4*, 0.98±0.08 *vs.* 0.98±0.09, $p=0.832$; *HPRT1*, 1.02±0.13 *vs.* 1.20±0.09, $p=0.203$) or the combination of two genes (*GUSB*, 1.00±0.04 *vs.* 0.93±0.06, $p=0.222$; *ATP2B4*, 1.01±0.02 *vs.* 1.07±0.07, $p=0.289$; *HPRT1*, 1.03±0.20 *vs.* 1.32±0.14, $p=0.203$).

To verify the discrepancies in gene stability found using NormFinder and geNorm, a second study was conducted with a greater number of samples, *CFTR*-wt controls (n=21) and *CFTR*-splicing group (n=18). Differences in raw data between both groups were again not statistically significant: *GUSB,* $p=0.791$; *ATP2B4*, $p=0.728$; *HPRT1*, $p=0.666$ (Fig. 1).

Although NormFinder analysis again considered *HPRT1* stable (0.006), it ranked *GUSB* as the most stable gene (0.003) and identified *GUSB* and *ATP2B4* as the best combination of two genes for normalization (0.003). The corresponding geNorm analysis showed the following values for each gene: *HPRT1*, M=0.689 and CV=0.376; *GUSB*, M=0.537 and CV=0.220; and *ATP2B4*, M=0.515 and CV=0.193, once more indicating the combination of the expression of the three genes was unstable. However, similarly to the first study, after *HPRT1* exclusion, *GUSB* (M=0.363, CV=0.127) and *ATP2B4* (M=0.363, CV=0.125) were considered suitable as normalizers.

The Mann–Whitney test again showed no statistically significant differences between groups in this second study, either for data normalized with the combination of the three genes (*GUSB*, 0.95±0.09 *vs.* 0.99±0.14, $p=0.662$; *ATP2B4*, 0.98±0.08 *vs.* 0.98±0.11, $p=0.535$; *HPRT1*, 1.03±0.07 *vs.* 1.08±0.13 $p=0.833$) or when data were normalized using the two gene combination (*GUSB*, 0.99±0.07 *vs.* 1.01±0.07, $p=0.573$; *ATP2B4*, 1.01±0.06 *vs.* 1.02±0.07, $p=0.526$; *HPRT1*, 1.05±0.10 *vs.* 1.13±0.20, $p=0.866$) (Fig. 2).

Overall, the second study supported the combination of *GUSB* and *ATP2B4* as a suitable normalizer for *CFTR* gene expression in NE.

Table 2
Calibration curve intervals and efficiencies of all genes analyzed in the three cell types.

Sample	*CFTR*		*GUSB*		*ATP2B4*		*HPRT1*		*B2M*	
	Interval	Efficiency	Interval	Efficiency	Interval	Efficiency	Interval	Efficiency	Interval	Efficiency
HEK 293 [a]	16.8–24.6	1.879	22.2–29.6	1.867	25.1–32.9	1.853	20.3–28.2	1.864	15.2–25.1	1.861
NP	26.4–34.5	1.875	22.1–31.8	1.901	23.1–31.1	1.893	20.3–30.1	1.857	16.0–26.4	1.859
NE	28.4–36.3	1.883	23.9–31.8	1.884	23.5–31.2	1.840	25.9–33.7	1.864	nd	nd

NP, nasal polyp; NE, nasal epithelium (pool of control samples); nd, not determined.
Expression interval data expressed as Cq values.
Efficiency value was obtained from each calibration curve (maximum efficiency value=2).
[a] HEK 293 cell line over-expressing wild type *CFTR* cDNA.

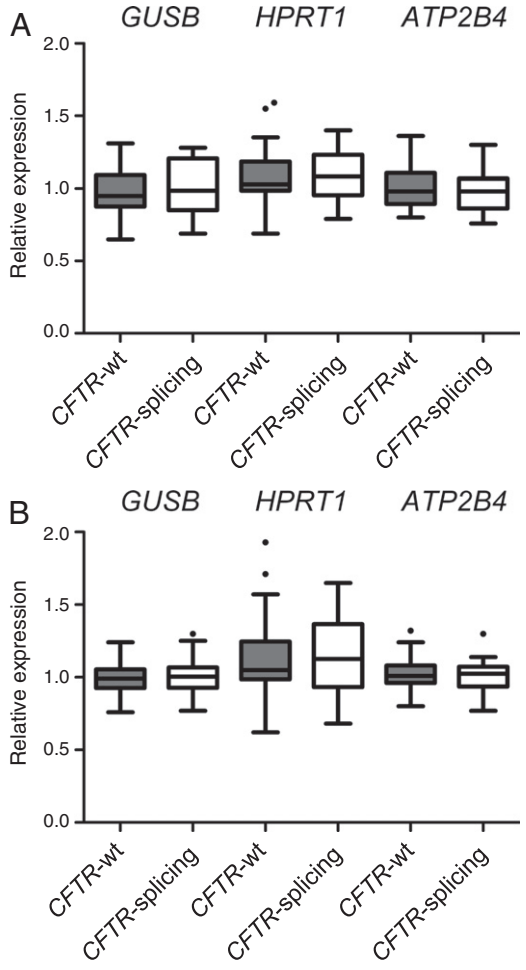

Fig. 2. Boxplot representation of reference gene expression variability for each individual group in the second study for the three genes tested. Data normalization was performed using a combination of three, *GUSB, HPRT1* and *ATP2B4* (A) and two, *GUSB* and *ATP2B4*, reference genes (B). Statistical significant differences were not found among the level expression in both groups. On the Y-axis gene expression is plotted. CNRQ median values and absolute deviation of the median (MAD) are also shown. Statistical analysis was performed using the Mann–Whitney test, considering $p<0.05$ statistically significant.

4. Discussion

Relative quantification of transcript levels by RT-qPCR relies on the simultaneous analysis of target and reference genes to provide an accurate interpretation of results. Commonly used reference genes (*GAPDH*, *ACTB*, *TPB*, *18s* among others) have often been adopted without assessing their specific tissue dependent behavior or the study design [9,20,21]. Once evaluated, some of these reference genes have shown variable expression in different tissues and/or under different experimental conditions [22,23]. Furthermore, most of them have shown a higher expression than the target gene [11], which is responsible for a significant bias concerning the interpretation of results of the target gene. In addition, several authors have demonstrated that the greater the number of reference genes used, the more accurately transcript amount of a target gene will be determined [9]. Therefore, validation of reference genes has become a pre-requisite for reliable data normalization for gene expression studies and the inclusion of more than one reference gene is strongly recommended whenever possible. Interestingly, guidelines for RT-qPCR gene expression analysis have been published recently to highlight the different variables involved and to show how to minimize their effect to improve accuracy and reproducibility [7].

So far, major attention has been focused on reliable reference genes to determine the profile of gene expression in tumor tissues [19,24–26]. However, to our knowledge this is the first study to evaluate reference genes for *CFTR* gene expression quantification. Taking into account that gene expression varies among the different tissues in an individual, we have developed this study using NE samples due to the accessibility of this sample commonly used in *CFTR* transcript analysis. The aim of this work was to validate reference genes for *CFTR* gene qPCR expression studies in NE tissue. To that end, the expression of four candidate reference genes: *B2M*, *GUSB, HPRT1* and *ATP2B4*, has been analyzed.

For the present analytical strategy many factors that could influence the final interpretation of results have been taken into account to obtain homogeneous raw data. First, to reduce the variability due to mRNA quality, NE brushing samples were collected in the same CF Unit and treated equally along all the steps of the process. The inclusion criteria applied also contributed to the reduction of variability between samples and its effect on results. Particularly, we included a RIN>5, without significant differences between the two *CFTR* groups as an indicator of similar RNA quality for downstream applications [8]. Secondly, to minimize the RT efficiency variation, all samples were processed simultaneously in two independent RT experiments.

Additionally, following guidelines for qPCR assays [7], the study has comprised two independent RT/sample, triplicate PCR reactions of each sample/gene, creation of calibration curves and the inclusion of controls to validate the experiments. The low *CFTR* gene expression in NE has been a key to create calibration curves with only four dilution points taking into account that a Cq above cycle 35 lacks reliability [27]. PCR efficiency and gene expression estimation were measured from calibration curves. In addition, the accuracy of the experiments is supported by the raw Cq analysis and the inter- and intra-assays CV (see Material and methods).

As expected, nasal samples (NP, NE) showed a significantly lower *CFTR* gene expression level compared with the HEK 293-*CFTR* (Table 2). However, the high sensitivity of RT-qPCR makes it able to reliably quantify the expression of genes with a low transcript level. Minor differences were found between NP and NE, supporting the suggestion that tissue specific expression must be considered.

The first decision concerning reference genes was to rule out the *B2M* as a candidate for normalization for *CFTR* gene expression considering that its higher expression level (Table 2) could lead to an erroneous interpretation of data of our target gene as stated in the guidelines [7].

For the remaining candidate genes, as previously suggested [28,29], several programs were applied to evaluate the

expression stability of reference genes in the two studies carried out (n=16 and n=39). The result of the expression stability obtained from the geNorm software was independent of the number of samples; both studies indicating the combination of *GUSB* and *ATP2B4* gene expression was suitable for data normalization. Independently, the NormFinder program changed the gene ranking depending on the number of samples, validating the same combination as geNorm once the number of samples had been increased (second study).

Furthermore, the Mann–Whitney analysis showed no statistical differences in the expression levels of candidate genes between groups in either of the normalized studies, or in raw data assessment, thus supporting their suitability as reference genes (Fig. 2). However, comparing both analyses, a lower dispersion was observed when data normalization was performed using the best two in the ranking, reinforcing *GUSB* and *ATP2B4* as the best combination for gene expression analysis in NE cells. This work does not rule out other putative reference genes not tested here.

On the other hand, the study has demonstrated that, although NE brushing provides a limited number of cells, this amount is enough for simultaneous gene expression analysis of the *CFTR* target and the two reference genes in duplicate.

In summary, we have developed the first validation study to identify suitable reference genes for data normalization for *CFTR* gene expression in NE cells by RT-qPCR. The present study shows that *the GUSB* and *ATP2B4* combination fulfills the stability criteria and similar level expression between the analyzed groups and accordingly, data normalization for *CFTR* gene expression can be reliably determined using a combination of these two reference genes. A main application of this study concerns *CFTR* mutations affecting transcript amount as well as therapies focused on this kind of mutations. Undoubtedly, the *ex vivo* analysis of mRNA by RT-qPCR will contribute to a better understanding of the relationship between *CFTR* transcript quantity and its clinical outcomes in the different affected tissues.

Acknowledgments

We are grateful to Dr. Rommens (Hospital for Sick Children, Toronto) for kindly providing the pCMVCFTRNot6.2 expression vector carrying 6.2 kb human wt *CFTR* cDNA. We thank Dr. Begoña Hurtado for her expert opinion. We also thank all participating subjects. S.L. is sponsored by the Researchers Stabilization Program from the SNS (CES09/020). This work was supported by ISCiii FIS/FEDER PI050804 and PI080041 and by Fundación Sira Carrasco (Spain).

The authors state that there is no conflict of interest.

References

[1] Castellani C, Cuppens H, Macek Jr M, et al. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. J Cyst Fibros 2008;7(3):179–96.

[2] Larriba S, Bassas L, Gimenez J, et al. Testicular CFTR splice variants in patients with congenital absence of the vas deferens. Hum Mol Genet 1998;7(11):1739–43.

[3] Chiba-Falek O, Parad RB, Kerem E, Kerem B. Variable levels of normal RNA in different fetal organs carrying a cystic fibrosis transmembrane conductance regulator splicing mutation. Am J Respir Crit Care Med 1999;159(6):1998–2002.

[4] Andrieux J, Audrezet MP, Frachon I, et al. Quantification of CFTR splice variants in adults with disseminated bronchiectasis, using the TaqMan fluorogenic detection system. Clin Genet 2002;62(1):60–7.

[5] Ramalho AS, Beck S, Meyer M, Penque D, Cutting GR, Amaral MD. Five percent of normal cystic fibrosis transmembrane conductance regulator mRNA ameliorates the severity of pulmonary disease in cystic fibrosis. Am J Respir Cell Mol Biol 2002;27(5):619–27.

[6] Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 2002;30(6): 503–12.

[7] Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22.

[8] Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 2006;27(2–3):126–39.

[9] Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7) [RESEARCH0034].

[10] Aerts JL, Gonzales MI, Topalian SL. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. Biotechniques 2004;36(1):84–91.

[11] Hruz T, Wyss M, Docquier M, et al. RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genomics 2011;12:156–69.

[12] Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 2005;6(4):279–84.

[13] Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004;37(1):112–4, 116, 118–9.

[14] Dheda K, Huggett JF, Chang JS, et al. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem 2005;344(1):141–3.

[15] Gene GG, Llobet A, Larriba S, et al. N-terminal CFTR missense variants severely affect the behavior of the CFTR chloride channel. Hum Mutat 2008;29(5):738–49.

[16] Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7:3.

[17] Calcagno AM, Chewning KJ, Wu CP, Ambudkar SV. Plasma membrane calcium ATPase (PMCA4): a housekeeper for RT-PCR relative quantification of polytopic membrane proteins. BMC Mol Biol 2006;7:29.

[18] Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007;8(2):R19.

[19] Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004;64(15):5245–50.

[20] de Kok JB, Roelofs RW, Giesendorf BA, et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest 2005;85(1):154–9.

[21] Hsiao LL, Dangond F, Yoshida T, et al. A compendium of gene expression in normal human tissues. Physiol Genomics 2001;7(2):97–104.

[22] Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004;15(3):155–66.

[23] Tricarico C, Pinzani P, Bianchi S, et al. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002;309(2):293–300.

[24] Coulson DT, Brockbank S, Quinn JG, et al. Identification of valid reference genes for the normalization of RT qPCR gene expression data in human brain tissue. BMC Mol Biol 2008;9:46.

[25] Fu LY, Jia HL, Dong QZ, et al. Suitable reference genes for real-time PCR in human HBV-related hepatocellular carcinoma with different clinical prognoses. BMC Cancer 2009;9:49.
[26] Fassunke J, Blum MC, Schildhaus HU, et al. qPCR in gastrointestinal stromal tumors: Evaluation of reference genes and expression analysis of KIT and the alternative receptor tyrosine kinases FLT3, CSF1-R, PDGFRB. MET and AXL BMC Mol Biol 2010;11:100.
[27] Applied-Biosystems. ABI Prism Bulletin #2; 2001.
[28] Willems E, Leyns L, Vandesompele J. Standardization of real-time PCR gene expression data from independent biological replicates. Anal Biochem 2008;379(1):127–9.
[29] Willems E, Mateizel I, Kemp C, Cauffman G, Sermon K, Leyns L. Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells. Int J Dev Biol 2006;50(7):627–35.